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(54) **Soluble interleukin-2 receptor as a disease indicator and a method of assaying the same.**

(57) The present invention discloses a sandwich method and a kit for assaying interleukin-2 receptor (IL-2R) in a sample. The method comprises determining reactivity of said sample with a plurality of ligands, each said ligand having binding affinity for a specific site on the receptor, said site being different

for each said ligand and distinct from interleukin-2 binding site on the receptor. The invention also discloses a method of detecting such disturbed or abnormal conditions in humans which release soluble IL-2R in the body fluid.

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ELISA ASSAY FOR SOLUBLE
HUMAN IL-2 RECEPTORS

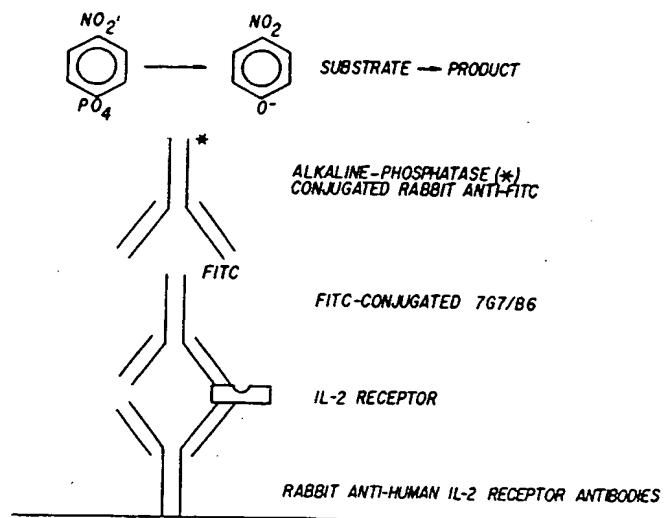


FIG. 1



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EUROPEAN SEARCH REPORT

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DOCUMENTS CONSIDERED TO BE RELEVANT		Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
Category	Citation of document with indication, where appropriate, of relevant passages		
A	FEDERATION PROCEEDINGS vol. 44, no. 4, 5 April 1985, Baltimore, MD, US; L.A. RUBIN et al.: "Soluble interleukin-2 receptors are shed by activated human lymphocytes in vitro" ---	1-11	G 01 N 33/68 C 12 P 21/02 G 01 N 33/543 G 01 N 33/566 G 01 N 33/577
P, X	HYBRIDOMA vol. 4, no. 2, April 1985, pages 91-102, New York, US; L.A. RUBIN et al.: "A Monoclonal Antibody, 7G7/B6, Binds to an Epitope on the Human Interleukin-2 (IL-2) Receptor That Is Distinct From That Recognized by IL-2 or Anti-Tac" * the whole document * ---	1-11	
D, A	NATURE vol. 311, 18 September 1984, pages 626-631, London, GB; W.J. LEONARD et al.: "Molecular cloning and expression of cDNAs for the human interleukin-2 receptor" * the whole document * ---	1-11	
D, A	SCIENCE vol. 224, 22 June 1984, pages 1312-1316, Washington, D.C., US; D.A. CANTRELL et al.: "The Interleukin-2 T-Cell System: A New Cell Growth Model" * the whole document *	1-11	G 01 N C 12 P
X, P	EP-A-0 162 699 (IMMUNEX CORPORATION) * pages 5-8 * ---	1-7	
E	EP-A-0 226 062 (BAYER AG) * the whole document * ---	1-19	
The present search report has been drawn up for all claims			
Place of search	Date of completion of the search	Examiner	
BERLIN	12-11-1992	DE KOK A.J.	
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone	T : theory or principle underlying the invention		
Y : particularly relevant if combined with another document of the same category	E : earlier patent document, but published on, or after the filing date		
A : technological background	D : document cited in the application		
O : non-written disclosure	I : document cited for other reasons		
P : intermediate document	& : member of the same patent family, corresponding document		



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Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
E	WO-A-8 705 912 (T CELL SCIENCES, INC.) * the whole document * -----	1-18	
TECHNICAL FIELDS SEARCHED (Int. Cl.5)			
The present search report has been drawn up for all claims			
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A : technological background	D : document cited in the application		
O : non-written disclosure	I : document cited for other reasons		
P : intermediate document	& : member of the same patent family, corresponding document		



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ELISA ASSAY FOR SOLUBLE
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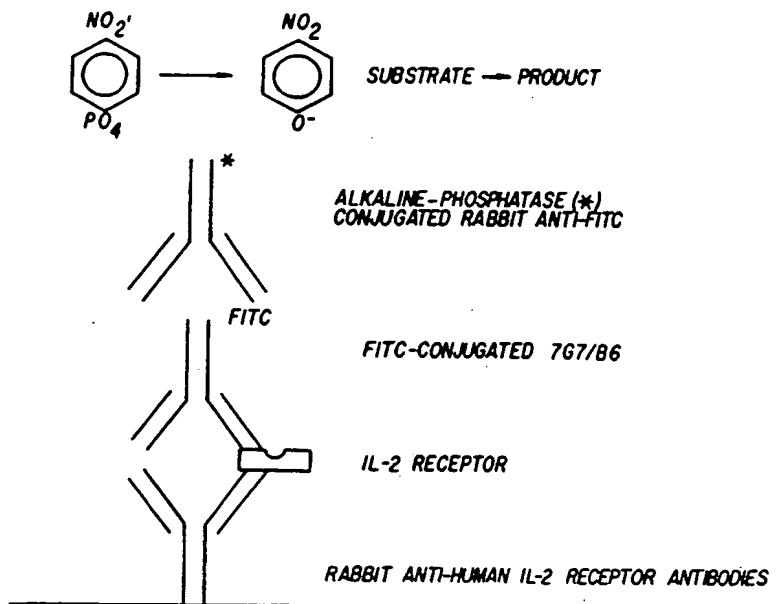


FIG. I

Technical Field:

The present invention is related to soluble interleukin-2 receptor (IL-2R) and a method of assaying the same. More particularly, the present invention is related to the discovery of soluble, IL-2R in human body fluid as a result of disturbed body function and a solid phase immunoassay for quantitative determination thereof.

Background Art

10 The ligand interleukin-2 (IL-2) and its receptor (IL-2R) *per se*, are well known entities. Interleukin-2 or T-cell growth factor as it is sometimes called, is a lymphokine, synthesized and secreted by lectin - or antigen-activated T-cells. The reported biological activities of IL-2 include stimulation of long-term *in vitro* growth of activated T-cell clones, induction of cytotoxic T-cell reactivity and the like. It is believed that IL-2 exerts its biological effects by interacting with specific high affinity receptors (IL-2R) on the surface of activated T-cells.

In contrast to hormone mediated systems, cellular activation is a prerequisite for the induction of both the ligand (IL-2) and its receptor (IL-2R), the latter being expressed rapidly at the cell surface in a time-dependent and heterogeneous manner (Central, et al, Science, 244:1312-1316, 1984).

When certain human cells are stimulated or otherwise activated, they manufacture and express newly synthesized molecules on their cell surfaces and also release these molecules in a soluble form. Hence, the detection of these molecules, which are not expressed by resting or unactivated cells, can serve as an indicator or "footprint" of cellular activation. As will be described more fully herein *infra*, it has been discovered that IL-2R is produced in the soluble form and circulated in the body fluid as a result of immune-activation or under certain malignant conditions particularly in lymphoreticular malignancies.

25 Heretofore, the measurement of IL-2 receptors (IL-2R) was limited to the assessment of cell-associated IL-2R by a variety of immunological techniques, involving several monoclonal antibodies against the human IL-2R. The observation of a soluble or released form of this molecule has heretofore not been reported. Therefore, this soluble molecule has never been measured in a body fluid. Hence, the detection of a soluble IL-2R molecule in humans under certain conditions is a new discovery.

30 With regard to cell-associated IL-2R, the quantitative measurement of IL-2R has been accomplished by binding of the monoclonal antibody followed by cellular analysis wth a fluorescence-activated cell sorter (FACS) or similar machine (costing approximately \$80,000-200,000). Such an analysis provides information about the number of cells expressing receptor and the amount of antibody bound, but does not provide a quantitative estimate of the number of receptors. The latter can be accomplished with an assay using 35 radiolabeled nonoclonal of known specific activity. However, such tests are tedious and require radionuclides which pose problems of safety and disposal.

In contrast, the Enzyme-Linked-Immunosorbent Assay (ELISA) of the present invention for IL-2R described in detail herein is quantitative, rapid, sensitive, inexpensive, and employs enzyme rather than radioactivity to detect IL-2R. The necessary reagents could be packed in a rather small box or kit and would 40 have a "shelf-life" of about one year. The instrument necessary to "read" the assay can be a simple spectrophotometer which is available in virtually every clinical hospital laboratory. In addition to the method described herein, this solid-phase immunoassay has also been performed with alkaline phosphatase conjugated monoclonal antibody (7G7/B6) directly, instead of the FITC-conjugated 7G7/B6 and alkaline phosphatase conjugated rabbit anti-FITC.

45 Disclosure of the Invention

It is, therefore, an object of the present invention to provide a quantitative, rapid, sensitive and inexpensive method of determining IL-2R.

50 It is a further object of the present invention to provide a solid phase immunoassay for quantitative determination of IL-2R.

It is a still further object of the present invention to provide a monoclonal antibody having specific binding affinity for an epitope of IL-2R, said epitope being different from IL-2 binding site and from a heteroantibody binding site of the receptor.

55 It is yet another object of the present invention to provide a "sandwich" Enzyme-Linked-Immunosorbent Assay (ELISA) for IL-2R.

An additional object of the present invention is to provide a method for monitoring, detecting or screening such malignant, therapeutic, autoimmune, or pathophysiological states in humans of which

soluble IL-2R level in the body fluid reflects an indicative index.

A still further object of the present invention is to provide a kit containing various components of the present sandwich ELISA for detecting soluble IL-2R, particularly in human plasma or serum.

Other objects and advantages will become apparent as the detailed description of the present invention proceeds.

Brief Description of the Drawings

These and other objects, features and many of the attendant advantages of the invention will be better understood upon a reading of the following detailed description when considered in connection with the accompanying drawings wherein:

Fig. 1 schematically shows various steps of the ELISA for soluble human IL-2R;

Fig. 2 shows serum IL-2R levels in normals and patients with several malignant diseases.

Fig. 3 shows sensitivity of the ELISA for detecting cellular and supernatant IL-2R. An IL-2 dependent T-cell line was stimulated with IL-2 and the culture supernatant (●) and detergent solubilized cells (■) analyzed by ELISA. Absorbance at 405 nm was related to the number of cells from which the cell extract and supernatant were derived;

Fig. 4 shows activating stimuli and the kinetics of cellular (A) and supernatant (B) IL-2R generation. Peripheral blood mononuclear cells (PBMC) were cultured *in vitro* with media (○), PHA (●), OKT3 (△), tetanus toxoid (■), or allogeneic cells (♦) for various times and the supernatant and cellular IL-2R determined. Results represent the units of IL-2R produced per 10^6 cultured PBMC;

Fig. 5 shows that soluble IL-2R production requires *de novo* protein synthesis but not cellular proliferation. Peripheral blood mononuclear cells were stimulated with PHA with no treatment (●), following irradiation with 3000R (♦) or in the presence of 25 g/ml cycloheximide (■). Both cellular (A) and supernatant (B) IL-2R were determined. Results represent the units of IL-2R produced per 10^6 cultured PBMC;

Fig. 6 shows molecular weight estimation of soluble IL-2R by Sephadex G-20 gel filtration. Concentrated supernatant from an IL-2 dependent T-cell line was sieved on a calibrated Sephadex G-200 column and the amount of IL-2 in the fractions determined by ELISA; and

Fig. 7 shows molecular weight estimation of soluble IL-2 by NaDODSO₄-PAGE. Supernatant IL-2 from HUT 102B2 was first passed over a normal mouse IgG2a Sepharose 4B column and then over a monoclonal anti-IL-2 (7G7/B6) Sepharose 4B column. The 7G7/B6 column was extensively washed with phosphate buffered saline and then eluted with 10 ml of 0.2 M glycine HCl. The eluted protein was lyophilized, resuspended in 300 μl of distilled water, and 25 μl analyzed on a 12.5% NaDODSO₄ polyacrylamide gel under reducing conditions, followed by silver staining. (Molecular weight markers: ovalbumin--43,000; carbonic anhydrase--30,000.)

Best Mode for Carrying out the Invention

These and other objects of the present invention are achieved by an inexpensive, rapid, sensitive and quantitative assay for detecting the presence of soluble IL-2 receptor in a sample, comprising a monoclonal antibody having specific binding affinity for an epitope of the receptor, said epitope being different from IL-2 binding site and from a heteroantibody binding site of the receptor.

Although any materials and methods similar or equivalent to the materials and methods described hereunder can be used in the testing or practice of the present invention, the preferred materials and methods are now described. All publications mentioned hereunder are incorporated herein by reference.

Materials and Methods

Source of Normal Cells and Cell Lines: Peripheral blood mononuclear cells (PBMC) were prepared from normal heparinized venous blood following standard procedure well known in the art. IL-2 dependent cell lines were made and maintained as described infra. The HTLV positive T-cell line HUT 102B2 has been well characterized (Poeisz, et al, *Nature* 294, 268-271, 1981) and was provided by Dr. N. Goldman, NIH, Bethesda, MD. The T-cell lines MOLT-4, CEM, and JURKAT (Uchiyama, et al, *J. Immunol.* 126, 1393-1397, 1981 and Robb, et al, *J. Exp. Med.* 160, 1126-1146, 1984), as well as the human Epstein-Barr virus (EBV) transformed B-lymphoblastoid lines were provided by Dr. R. Quinones, NIH, Bethesda, MD. The pre B-cell lines NALM-1 and NALL-1 (Korsmeyer, et al, *J. Clin. Invest.* 71, 301-313, 1983), and the B-cell lines NU-DHL1 and SU-DHL6 (Winter, et al, *Blood* 63, 140-146, 1984) were obtained from Dr. S. Korsmeyer of NIH,

Bethesda, MD. A second monoclonal antibody against the IL-2R, termed anti-Tac as described by Uchiyama, et al, supra, was obtained from Dr. T. Waldmann, NIH, Bethesda, MD.

Culture Stimulants: Phytohaemagglutinin (PHA/GIBCO) was used at a final dilution of 1/200 (v/v), purified OKT-3 at 10 ng/ml, Tetanus toxoid (Massachusetts Dept. of Public Health) at a final concentration of 6 Lf Units/ml, and Pokeweed mitogen (PWM/Gibco) at a final dilution of 1/100 (v:v).

Cells and Cell Cultures: PHA activated lymphoblasts from normal individuals were produced in bulk culture by adding 10 ml of peripheral blood mononuclear cells (PBMC) at 1×10^6 cells/ml suspended in 1640 media containing 5% heat-inactivated (56° C, 30 minutes) fetal calf serum (FCS) (Gibco) to tissue flasks (Costar #3275, Cambridge, MA) with PHA (Gibco) at a final dilution of 1/200 (v/v), and incubated for 72 hours in a humidified 5% CO₂ in air atmosphere at 37° C. Concanavalin A (Con A) (Sigma Chemicals, St. Louis, MO) stimulated PBMC were prepared identically with 10 µg/ml of Con A. B-cell lymphoblastoid lines were from normal PBMC transformed with the B95-8 strain of Epstein-Barr virus.

The HTLV-1 positive T-cell leukemia line, HUT 102B2 was maintained *in vitro* by serial passage in antibiotic supplemented RPMI 1640 containing 10% FCS and added Gentamycin (50 µg/ml, Schering Corp. Kenneworth, NJ). An IL-2 dependent T-cell line was prepared from 72 hr PHA lymphoblasts by resuspending the cells at 1×10^6 cells/ml in antibiotic supplemented 1640 media with 5% FCS and 10% (v/v) lectin-free IL-2 (Cellular Products, Buffalo, NY) and reculturing for two passages of 3-5 days each. The responsiveness of such cells to IL-2 and the inhibition of IL-2 induced proliferation by 7G7/B6 or anti-Tac was tested by incubating 50×10^3 cells in 200 µl of media in quadruplicate in the wells of 96 well microtitre plates (Costar #3596) for 72 hours without added IL-2, or with added IL-2 and equivalent concentrations of either 7G7/B6 or anti-Tac. Cultures were pulsed with 1 µ Ci of ³H-Thymidine (New England Nuclear, Boston, MA, 20 Ci/mmol) for the final 4 hours of culture. The assessment of the proliferative and cytotoxic responses of mixed leucocyte cultures (MLC) in the presence or absence of 7G7/B6 were performed as described by Uchiyama, et al, J. Immunol. 126:1398, 1981. In addition, untreated allostimulated cells were coated with 7G7/B6 or anti-Tac and separated into positive and negative populations employing Sephadex G-200 anti-mouse Fab columns as described by Uchiyama, et al, supra. These separated cells were then tested for cytotoxic effector activity as described by Uchiyama, et al, supra. T-cells and non T-cells were separated by AET treated sheep erythrocyte rosetting as described by Tosato, et al NEJM 301:1133, 1979.

Stimulated Cultures: Cultures stimulated with PHA or OKT-3 contained 5×10^5 cells in 2 mls of RPMI 1640 with 10% heat inactivated fetal calf serum (FCS) supplemented with Penicillin 100 U/ml and Streptomycin 100 µg/ml. Cultures stimulated with PWM contained 2×10^6 cells in 2 mls of the same medium. Cultures stimulated with Tetanus toxoid or allogeneic cells contained 3×10^6 and 2×10^6 cells/culture, respectively, in 2 mls of the same media containing 10% pooled human plasma. Allogeneic cultures consisted of 1×10^6 responder PBMC and 1×10^6 irradiated (3000R) stimulator PBMC. Triplicate replicate cultures were established in 24-well plates (Costar, Cambridge, MA) and incubated at 37° C in a humidified 5% CO₂ and air atmosphere. The cultures were harvested at various times, the replicates pooled and centrifuged (400xGx10mins.) to separate the cells and supernatant. The proteinase inhibitors phenylmethyl sulfonyl-fluoride (2mM) and tosylamide-2-phenylethylchloromethyl ketone (150 µg/ml) were added to the supernatants. After washing, the cells were lysed in 25 mM Tris-buffered saline pH 7.4 containing 1% Triton X-100 and proteinase inhibitors. Supernatants and cell extracts were stored at -20° C until use.

Monoclonal Antibodies: Production of the Monoclonal antibody 7G7/B6. BalbC/J mice (Jackson Labs. Bar Harbor, ME) were injected intraperitoneally and subcutaneously with 20×10^6 A/Hong Kong influenza virus stimulated PBMC on two occasions 30 days apart, followed by an injection of 14×10^6 cells intravenously one month later. One week after the third injection, immune splenocytes were fused with SP2/0 cells by the method of Ozato, et al, J. Immunol. 124:533, 1980. Hybridoma culture supernatants, screened by complement mediated lysis which produced antibodies which bound to phytohaemagglutinin (PHA) stimulated lymphoblasts but not B-lymphoblastoid cells from the donor of the influenza-stimulated PBMC, were selected and cloned in microtiter wells on a feeder layer of rat fibroblasts at 0.3 cells/well. One rapidly growing clone which produced large amounts of an IgG2a antibody was selected and designated 7G7/B6. The hybridoma which produces this monoclonal antibody (7G7/B6) has been deposited in the American Type Culture Collection, Rockville, MD under Accession No. HB 8784. A monoclonal anti-HLA-A and -B framework antibody of IgG2a subclass was purchased from Bethesda Research Laboratories, Gaithersburg, MD. Partially purified 7G7/B6 were prepared from ascites by chromatography on Sépharose 4B bound *Staphylococcus* protein A (Pharmacia Chemicals, Piscataway, NJ), as described by Ey, et al, Immunochem. 15:429, 1978. Rabbit anti-human IL-2R heteroantibodies are produced following standard procedure using purified IL-2R as an immunogen (Leonard, et al, Nature, 311:626-631, 1984).

Fluorescence Activated Cell Sorter (FACS) Analysis

Cytofluorometric analysis of the binding pattern of 7B7/B6 to both resting and activated PBMC was done by indirect immunofluorescence utilizing a fluorescein isothiocyanate (FITC) conjugated F(ab')2-fragment of a goat anti-mouse IgG antibody (N.L. Cappell Labs, Cochranville, PA) following routine procedure.

5 The competitive binding of 7G7/B6 or anti-Tac to activated cells was assessed using biotinylated forms of the monoclonal antibody as described by Titus, et al, J. Immunol. Methods, 50:193, 1982. PHA activated PBMC were first incubated at 4°C with unlabelled 7G7/B6 or anti-Tac at a known saturating dose, washed with cold media, and incubated with the biotinylated forms of either the same or alternate monoclonal antibody for 30 minutes at 4°C. The cells were washed with cold media and incubated with 25 µl of a 1/40 dilution of FITC modified Avidin (Sigma) for 30 mins. at 4°C, washed twice and analyzed on the FACS.

10 The ability of IL-2 to block the binding of antibodies to activated cells was assessed by incubating 1x10⁶ PHA activated PBMC for 1 hour at 4°C in a final volume of 100 µl of various concentrations of immunoaffinity purified IL-2 (Robb, et al, Proc, National Academy of Sciences, USA, 80:5990, 1983). Subsaturating doses of biotinylated antibodies were added. The cells were incubated for 30 mins. at 4°C, followed by two washes in cold media, stained with FITC Avidin as above and analyzed on the FACS.

Cell Radiolabelling and Immunoprecipitation Experiments:

125₁ Cell Surface Labeling: Tris buffered Saline (TBS) was prepared with 25 mM Tris (BRL) in 20 Dulbecco's PBS (Gibco) at pH 7.4 to which 2 mM phenylmethylsulfonyl fluoride, and 150 µg/ml of L-1-tosylamide-2phenylethylchloro-methyl ketone (Sigma) were added. Lysi buffer consisted of TBS with 1% Triton X-100 (RPI Elk Grove, IL). About one hundred million PHA activated PBMC or HUT 102B2 cells were washed, resuspended in 1 ml of PBS and surface radiolabelled with 10 µl of ¹²⁵I (Na salt, 100 mCi/ml, Amersham, Arlington Hts., IL) by lactoperoxidase (Sigma) catalyzed iodination as described by Ledbetter et 25 al, J. Exp. Med. 153:310, 1981. Labeled cells were solubilized at a concentration of 10x10⁶ cells/ml with lysis buffer and the supernatant, after centrifugation, was retained. 1 ml aliquots of the supernatant were incubated with 2 µg of 7G7/B6, or anti-HLA for 2 hours at 4°C followed by a 1 hour incubation at 4°C with 30 washed 10% Staphylococcus aureus (Cowan, strain 1, ATCC 12598, Rockville, MD). The suspensions were centrifuged and the immunoprecipitates washed 5 times, and then boiled for 2 mins. in 25 µl of 62.5 mM Tris HCL with 2% sodium dodecyl sulphate (SDS), 10% glycerol, 5% beta-mercaptoethanol, and 0.05% bromophenol blue (all from Sigma). After pelleting the staphylococci, the supernatants were removed and electrophoresed on a 12.5% SDS polyacrylamide gel followed by autoradiographic analysis per standard procedures.

³⁵S-Methionine Pulse-Chase Labeling:

About hundred million HUT 102B2 cells were washed once in methionine free RPMI 1640 (NIH media unit) containing 2% dialyzed FCS and resuspended at 10x10⁶ cells/ml in the same media for 1 hour at 37°C. The cells were pelleted, resuspended at 1x10⁶ cells/ml in the same media and pulsed with 250 µ Ci/ml of ³⁵S-methionine (1315 Ci/mmol, Amersham) for 30 mins. The cells were pelleted and diluted to 2x10⁶ cells/ml in RPMI 1640 containing excess (500 fold) unlabeled methionine (Gibco). Incubation of the cultures was continued and at intervals of 1,2,4,8, and 22 hours aliquots of 2x10⁷ cells were removed and diluted in a 10 fold volume of 4°C PBS containing 50 µg/ml of cycloheximide (Sigma). Each aliquot was pelleted and lysed in the same manner as those cells undergoing surface radiolabelling. Prior to immunoprecipitation with 7G7/B6 or anti-Tac samples were first precleared with anti-HLA. Anti-HLA, 7G7/B6 and anti-Tac immunoprecipitates were analyzed on SDS polyacrylamide gels as above.

Cross Clearing Studies: Sequential immunoprecipitations were performed on both surfaces as well as biosynthetically labeled HUT 102B2 cells. Aliquots were prepared as described above and immunoprecipitated first with anti-HLA, or 7G7/B6 or anti-Tac. The supernatants from these immunoprecipitates were removed and then immunoprecipitated with either the same or alternate monoclonal antibody (Mab). Optimal doses of the Mab were used in these studies for preabsorption and subsequent immunoprecipitation to ensure the identification of any remaining protein which might be precipitated by the Mab. For the sequential immunoprecipitation studies of the biosynthetically labeled cells, experiments were performed at both an early (<30 mins.) and late (>8 hours) time point of a pulse chase study.

Assay for Soluble IL-2 Receptor (IL-2R):

For the ELISA to detect soluble IL-2R, alternate rows of the inner 60 wells of Immulon-1 flat-bottomed

96 well microtiter plates (Dynatech) were coated overnight with 150 μ l of purified anti-Tac at 1 μ l/ml in carbonate buffer pH 9.6 or carbonate buffer alone as a background control. It should be noted here that a heteroantibody, such as a rabbit anti-IL2 receptor antibody, can also be used in place of anti-Tac. In fact, the use of heteroantibody provides a more comprehensive and desirable assay. After washing, 100 μ l of various dilutions of samples was added to the coated and control wells, incubated for 2 hours, washed, and 100 μ l of a 1/4,000 dilution of FITC-conjugated 7G7/B6 in phosphate buffered saline containing 1% FCS added to all the wells. After two hours additional incubation, the plates were washed and 100 μ l of a 1/1,000 dilution of alkaline phosphatase conjugated rabbit anti-FITC was added. After a one hour incubation, the plates were washed, 100 μ l of p-nitrophenyl phosphate (Sigma/1 mg/ml) added, and the absorbance of the wells determined after 30 mins. at 405 nM using a Titertek ELISA reader (Flow Laboratories). The ELISA for IgM was performed as described by Yarchoan, et al, J. Immunol. 127, 1958-1964, 1981. The specificity of the ELISA for soluble IL-2R is determined by coating plates with buffer alone, anti-IgM, a monoclonal or polyclonal antibody. These wells then received the day 12 supernatant of PWM activated PBMC, followed by either FITC conjugated anti-IgM (Yarchoan, et al. Immunol. 132, 2696-22699, 1984) or alternatively FITC conjugated 7G7/B6 (Rubin et al, Hybridoma, 1985), and finally rabbit anti-FITC as described supra.

Alternative Radioisotopic Method for IL-2

Method: The wells of a polyvinyl 96 well microtiter plate (Cooke) were coated overnight with 150 μ l of a solution of the monoclonal antibody 7G7/B6 suspended in carbonate buffer pH 9.6 at 1 μ g/ml or 150 μ l of carbonate buffer alone. The wells of the plate were washed and 100 μ l of various concentrations of detergent solubilized cell extracts or cell-free culture supernatant from the same cells were added to the coated and non-coated (control wells) in triplicate. In this experiment, tonsillar mononuclear cells were activated for 72 hours *in vitro* with phytohemagglutinin. Following a two hour incubation at room temperature, the plates were washed with buffer. (The buffers and diluents employed are the same as those employed for the sandwich ELISA.) Each well then received 20,510 cpm of tritium-labelled anti-Tac prepared as described (Leonard, et al, Proc. Nat. Acad. Sci. 80, 6957-6961, 1983) in a volume of 100 μ l. After an additional two hour incubation, the plates were washed, dried, and individual wells were cut from the plate. The wells were placed in scintillation vials along with 10 ml of scintillation fluid (Ultrafluor) and the radioactivity bound to the wells determined by liquid scintillationspectroscopy. In all cases the cpm bound to wells coated with carbonate buffer alone was less than 160 cpm.

Molecular Weight Estimation: Sephadex G-200 gel filtration was performed using the concentrated (10X) supernatant of an IL-2 dependent cell line. Following molecular sieving, the individual fractions (2.5 mls) were lyophilized, resuspended in 100 μ l of distilled water and assayed by ELISA to determine IL-2R content. Control and 7G7/B6 immunoaffinity columns were prepared by coupling either normal mouse IgG2a or purified 7G7/B6 to cyanogen bromide activated Sepharose 4B (Phramacia). 600 mls of HUT 102B2 culture supernatant was concentrated and passed first over a normal mouse IgG2a Sepharose 4B column followed by passage over 7G7/B6 Sepharose 4B column. The material bound to the latter column was eluted with a small volume of 0.2 M glycine-HCl pH 2.5, dialyzed against PBS, lyophilized, and resuspended in 300 μ l of distilled water. 25 μ l of this concentrated material was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Na-Dod-SO₄ PAGE).

ELISA Reactivity of Cell Associated and Supernatant IL-2R:

As shown in Fig. 3 the results indicated that IL-2R was measurable in cell-associated and cell-free supernatants from an IL-2 dependent human T-cell line stimulated with IL-2 at a cell concentration of 1×10^5 cells/ml and harvested four days later when the cell concentration was 1×10^6 cells/ml. Various dilutions of culture supernatant or detergent extracted cells were analyzed by ELISA and the absorbance at 405 nM plotted against the number of cells from which the supernatant and cell extract were derived. As shown in Fig. 3 both supernatant and cell associated IL-2R could be detected above background levels at cell numbers as low as 6×10^4 and 3×10^4 /ml, respectively. In order to ascertain that the supernatant material was indeed a soluble form of the IL-2R, this supernatant was passed thru a 0.22 micron Millipore filter and also ultracentrifuged for 100,000 x G for 1 hour. Neither procedure diminished the amount of IL-2R detected in the supernatant. To establish the specificity of the ELISA for soluble IL-2R, culture supernatants from PBMC activated for 12 days with PWM were analyzed for IL-2R and IgM. The data in Table 1 show that the absorbance in uncoated wells receiving sample and either FITC anti-IgM or FITC 7G7/B6 (second antibody) plus alkaline phosphatase conjugated anti-FITC (third antibody) was very low. Wells coated with heteroantibody which then received sample and FITC 7G7/B6 (but not FITC anti-IgM), followed by third antibody,

were strongly positive. Wells coated with anti-IgM which received sample, FITC anti-IgM (but not FITC 7G7/B6), and third antibody, were also strongly positive. Those wells that were coated with anti-IgM which received sample, FITC 7G7/B6, and third antibody or those wells coated with heteroantibody which received sample FITC anti-IgM, and third antibody had adsorbance comparable to uncoated wells. These results
5 establish that this assay is specific for the molecule bound by 7G7/B6 and heteroantibody.

Activating Stimuli and Kinetics of Cell-Associated and Soluble IL-2R Expression:

As shown in Fig. 4 A&B, supernatant and cell-associated IL-2R are generated early and with rapid
10 accumulation in those cultures stimulated with PHA or OKT3 while the increase in cultures stimulated with tetanus toxoid and allogeneic cells was more gradual. In addition, cellular and soluble IL-2R generated in response to antigen activation at the time points tested was somewhat less than that produced in response to PHA and OKT3 stimulation.

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Table 1

Coating antibody	Sample	Second antibody	Third antibody	Absorbance
None	PWM sup.	FITC anti-IgM FITC 7G7/B6	Anti-FITC "	0.036 0.004
None	"	FITC 7G7/B6	"	
anti-Tac	"	FITC anti-IgM	"	2.442
anti-Tac	"	FITC anti-IgM	"	0.029
anti-IgM	"	FITC 7G7/B6	"	2.346
anti-IgM	"			0.004

Legend Table 1

Specificity of an ELISA for soluble IL-2R. Supernatants were obtained from PWM stimulated PBMC at 12 days of culture. Wells coated with either buffer alone, anti-Tac or anti-IgM received PWM supernatant, followed by either FITC 7G7/B6 or FITC anti-IgM in the combinations shown. All wells then received alkaline phosphatase conjugated anti-FITC. P-nitrophenyl phosphate substrate was added and the absorbance measured at 405 nM.

5 Cultures stimulated with media alone generated essentially no measurable levels of IL-2R. Cellular IL-2R in cultures stimulated with PHA and OKT3 declined from peak levels on day 7 of culture while supernatant IL-2R continued to rise. To determine if the soluble IL-2R was the result of cell death and subsequent release of cell associated IL-2R, cultures of PBMC stimulated for 24 hours with PHA which possessed 500 cell-associated IL-2R units per 10^6 cells were washed, either sham treated or repetitively frozen and thawed and placed back in culture in the same supernatant in which they had been generated. The data in Table 2 show that there was no additional accumulation of soluble IL-2R in the supernatant from the killed cells, while the sham treated cells produced soluble IL-2R in the supernatant.

10

Supernatant IL-2R Generation is Radioresistant but Requires Protein Synthesis:

15 Since cell-associated IL-2R expression has been shown to require *de novo* protein synthesis but not cellular proliferation, (Uchiyama, et al, supra) the effect of irradiation and of polypeptide synthesis inhibitor cycloheximide on supernatant IL-2R was examined. Figs. 5, A&B show that irradiation with 3000R did not inhibit supernatant or cell-associated IL-2R generation while cycloheximide (25 μ g/ml) completely inhibited the generation of both forms of IL-2R.

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Table 2

Treatment	Supernatant IL-2R Levels on Day		
	1	2	3
None	187	674	1,265
Freeze/Thaw	187	195	158

Legend Table 2

Soluble IL-2R is not released from killed IL-2R positive cells in vitro. Normal PBMC were activated with PHA for 24 hours and the quantity of soluble and cellular IL-2R determined by ELISA. These activated cells were then subjected to three cycles of freezing and thawing or sham treated and returned to the same IL-2 and IL-2R containing media for additional periods of in vitro culture. The level of supernatant IL-2R was determined by ELISA. Results represent the units of IL-2R per 10^6 cultured PBMC.

Analysis of Cell Lines for the Capacity to Release IL-2R:

5 Because HTLV I infected T-cell lines constitutively express cell surface IL-2R (Robb et al, J. Exp. Med. 160, 1126-1146, 1984), the cells and supernatants of several human tumor cell lines were examined for IL-
 10 2R. The data in Table 3 show that the HTLV I positive T-cell line HUT 102B2 expressed high levels of cellular associated IL-2R, and also released large amounts of IL-2R into supernatants compared to the levels of IL-
 15 2R expressed and released by an IL-2 dependent cell line. HTLV I negative T-cell lines such as MOLT 4, JURKAT, and CEM did not express cellular IL-2R nor release soluble IL-2R. The pre B-cell lines NALM-1 and NALL-1, and the B-cell lines NU-DHL1 and SU-DHL6, did not express or release IL-2R into culture supernatants. In contrast, all three EBV induced B-cell lines expressed low levels of cellular IL-2R and one of these lines released measurable IL-2R into the culture supernatant.

15 Molecular Weight Estimation of the Released Soluble IL-2R:

The soluble IL-2R from an IL-2 dependent T-cell line analyzed by Sephadex G-20 gel filtration (Fig. 6) appears to be relatively uniform in size with a molecular weight somewhat smaller than albumin. To better define the molecular weight of the soluble IL-2R, immunoaffinity purified soluble IL-2R from HUT 102B2
 20 cells was analyzed by NaDODSO4 PAGE. Fig. 7 shows that the soluble IL-2R migrated as a single broad band of apparent molecular weight Mr = 40,000.

Table 3

Cell Line	Cell Type	Cellular IL-2R	Supernatant IL-2R
IL-2 dependent line	T-cell	1,250	1,000
HUT 102B2	T-cell	4,800	11,000
CEM	T-cell	<31.3	<31.3
JURKAT	T-cell	<31.3	<31.3
MOLT-4	T-cell	<31.3	<31.3
NALM-1	pre-B-cell	<31.3	<31.3
NALL-1	pre-B-cell	<31.3	<31.3
EBV-1	B-cell	54.3	<31.3
EBV-2	B-cell	55.0	<31.3
EBV-3	B-cell	205.0	65.0
NU-DHL1	B-cell	<31.3	<31.3
BU-DHL6	B-cell	<31.3	<31.3

Legend Table 3

Analysis of Various Cell Lines for the presence of cellular and supernatant IL-2R. In vitro cultured cell lines were analyzed for the level of cellular and supernatant IL-2R receptors by ELISA. Results represent the units of IL-2R per 10^6 cultured cells.

45 The "Sandwich" ELISA:

The term "sandwich ELISA or "sandwich" assay as used herein means that at least two antibodies or reactive fragments thereof having different binding sites on IL-2R are used as if the receptor were sandwiched between these two antibodies. These antibodies could either be both monoclonal or one monoclonal and the other a heteroantibody (polyclonal) or both could be heteroantibody.

As shown in Fig. 1, rabbit anti-human IL-2 receptor antibody is diluted to a desired concentration (1 to 1,200) in carbonate buffer pH 9.6 and 150 μ l added to the wells of alternate columns of the inner 60 wells of Immulon-1 flat-bottomed 96-well microtiter plates (Dynatech). The alternate wells of the plate receive carbonate buffer alone. Following overnight or longer incubation at 4°C, the wells are washed with buffer (phosphate buffered saline pH 7.4 containing 0.05% tween and sodium azide), and various dilutions of the sample to be analyzed are applied to the coated and non-coated wells. The diluent consists of phosphate-buffered saline containing tween, azide, and 1% fetal calf serum or other protein source (diluent). A

reference preparation of soluble IL-2 receptors is also added to the coated and non-coated wells. Following a two hour incubation at room temperature, the plates are washed with buffer (PBS with tween and azide as above) and 100 μ l of a 1:1,000 dilution of fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody (7G7/B6) is added and the plates incubated for 2 hours at room temperature. Following this incubation, the 5 plates are washed and 100 μ l of alkaline phosphatase conjugated rabbit anti-FITC diluted 1:1,000 in diluent is added. After a 1 hour incubation, the plates are washed and 100 μ l of a solution of p-nitro phenylphosphate (PNPP, Sigma, 1 mg/ml) substrate in diethanolamine buffer pH 9.8 is added to the wells. The conversion of 10 substrate (PNPP) to produce (PNP) is determined by absorbance at 405 nM in the experimental (coated) and control (uncoated) wells and the latter subtracted from the experimental wells. The specific product produced in the experimental wells is then related to that produced in the standard wells and the amount of IL-2R present in an unknown sample is determined from the readings obtained for various known levels of IL-2R.

It should be noted that the preferred embodiment of the method described, *supra*, is in indirect or "amplified" Enzyme-Lined Immunosorbent Assay (ELISA). Of course, multiple modifications of the present 15 method can be devised by the artisan in accordance with the description outlined herein.

Some alternatives to the method include:

- (a) The soluble molecules (IL-2R) are measurable either using solid phase assays, fluid phase assays, or semi-solid phase assays (antibody or ligand in gel). The method need not be limited to only solid-phase systems as should be evident to one of ordinary skill in the art to which this invention pertains.
- 20 (b) In the case of solid phase assays, the soluble IL-2 receptor can be captured for analysis by materials binding either non-specifically or specifically to the receptor. To ensure specificity of the assay, however, the former must detect receptor by a specific means, e.g. antibody, IL-2, or other specific ligand. Such antibodies include, but are not limited to, heteroantisera (all various species) binding to the receptor, monoclonal heterologous and homologous antibodies (hybridoma derived or otherwise), and by specific 25 ligand (IL-2). In those cases where the receptor is captured using materials which are specific to the receptor including heterologous and homologous antisera, monoclonal antibodies (hybridoma derived or otherwise), and specific ligand (IL-R) binding to the receptor, a non-specific means of detecting the receptor can be employed such as heteroantisera containing antibodies to the human IL-2R as well as certain ligands binding to various epitopes on the molecule which are not specific to the molecule, e.g. 30 sugar residues and the like.
- (c) If captured on a solid surface for analysis, the solid surface may consist of polymeric compounds, glass or ceramic materials, inorganic matrices, agarose, magnetic beads, and other adsorbents well known in the art.
- 35 (d) The specific or non-specific materials binding to the receptor could be "tagged" with various enzymes (alkaline-phosphatase, glucose oxidase, peroxidase, and the like); radioisotopes (I^{125} , H^3 , C^{14} and the like) or other materials to allow the detection of binding. In addition, "competitive" assays could be established in which the binding of labeled ligand to the receptor is inhibited by the binding of "cold" or non-labeled ligand. It should be noted that in addition to the "indirect or amplified" assays, ligand 40 binding directly to the receptor could also be used for the detection of receptor. Furthermore, a plurality of layers of reagents could be used to "amplify" the assay.
- (e) It should be emphasized that the buffers, the incubation times, the substrate, the enzyme and the like mentioned here are only exemplary. Of course, any of the described incubation times the buffers employed for binding the antisera or ligand to the solid surface, the buffers used for washing, diluting, or substrate solubilization, and the wavelength of light used for determining the conversion of substrate to 45 product could be suitably modified. Any suitable substrate which will produce a product measurable at a wavelength different from that of the substrate can also be employed.
- (f) Another method to detect the receptor would be to allow binding of the specific ligand to the receptor then detect the presence of the ligand by antibodies to the ligand either by an indirect or direct method.
- 50 (g) If analyzed in the fluid phase any suitable solvent can be employed. In the fluid phase, the assay could be performed as a standard competitive radioimmunoassay involving radiolabeled IL-2 receptor, antibody to the IL-2 receptor (7G7/B6 or other antibodies), a ligand binding to the anti-receptor antibody but not to the IL-2 receptor and non-radiolabeled IL-2 receptor. In addition to the use of radioisotopically labeled IL-2R, the receptor could be labeled with enzyme or other materials to allow its detection. Other fluid phase assays could involve "tagged" IL-2 to detect the receptor in the fluid phase as well as salt or other methods to precipitate the receptor ligand complex.
- 55 (h) In addition to absorbance changes noted during the enzymatic conversion of substrate to product, this assay can also be conducted using radioisotopic means, for example as shown in Table 4. As can be seen, soluble IL-2 receptors can be measured by the binding of 3H -labeled monoclonal antibodies.

The data also show that IL-2R can be detected either in cell extracts or culture supernatants. In addition, the conversion of radioisotopic substrate to non-radioisotopic product can also be used. However, the non-radioactive, ELISA as described supra is the preferred method.

5
Table 4: A Solid Phase Radioisotopic Method
for the Measurement of
Soluble IL-2 Receptors

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Source of Soluble IL-2R	Units of IL-2R/ml#	CPM of Tritiated anti-IL-2R Bound
Detergent solubilized cells	24,800	6,956 x/ \pm 1.07*
" " "	12,400	7,886 x/ \pm 1.01
" " "	6,200	7,887 x/ \pm 1.01
" " "	3,100	5,459 x/ \pm 1.01
" " "	1,550	2,967 x/ \pm 1.01
Culture supernatant	4,600	5,059 x/ \pm 1.02
" " "	2,300	3,038 x/ \pm 1.03
" " "	1,150	1,706 x/ \pm 1.03
" " "	575	940 x/ \pm 1.07
" " "	288	597 x/ \pm 1.01
" " "	144	351 x/ \pm 1.04

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* Values represent the geometric mean counts per
minute.

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x/ \pm 1 standard error of the mean of Tritiated anti-Tac
bound to the plates.

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The concentration of soluble IL-2R in these
preparations in units/ml was determined by the
sandwich ELISA.

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The demonstration herein that the release or expression of IL-2R is associated with immune activation makes it now possible to monitor the cell-associated and soluble IL-2R in various body fluids as a sensitive test of altered states of immune activation, such as occurs in rheumatoid arthritis and systemic lupus erythematosus, for example. The ELISA test of the present invention can be valuable to predict when disease exacerbations are about to occur and/or in monitoring immunosuppressive therapy. In a similar way
45 determination of IL-2R in the serum of individuals rejecting allografted kidneys, livers and the like would allow monitoring graft rejection and titrating immunosuppressive therapy.

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The demonstration of elevated serum levels of IL-2R in association with certain malignancies as shown in Fig. 2, establishes that an analysis of cells and serum from certain patients with such cancers is a useful diagnostic means for detecting and/or monitoring initial or residual tumor burden.

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It should be noted that the present sensitive assay for IL-2R also enables the dosage of IL-2R administered to humans to be monitored much as the serum levels of pharmaceuticals are checked.

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Since the present test for soluble IL-2R requires hybridoma derived monoclonal antibodies which can be produced in large quantities, the quantities of reagents needed is no longer a problem. Mice (costing approximately \$10 each) injected with the hybridoma cells, would generate enough reagents for the performance of roughly 50,000 tests making it obviously quite inexpensive. The performance of this test as an Enzyme-Linked Immunosorbent Assay (ELISA) requires no radioisotopes and the reagents are stable over a period of about one year, which are significant advantages of the present invention.

It is understood that the examples and embodiments described herein are for illustrative purposes only

and the various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

Claims

- 5 1. A method for detecting the presence of interleukin-2 receptor in a sample comprising determining reactivity of said sample with a plurality of ligands, each said ligand having binding affinity for a specific site on the receptor, said site being different for each said ligand and at least one of said ligands being a monoclonal antibody derived from a hybridoma having American Type Culture Collection, Accession n° HB8784.
- 10 2. The method of claim 1 employing at least two ligands.
- 15 3. The method of claim 2 wherein a second ligand is a monoclonal antibody or a reactive fragment thereof.
- 20 4. The method of claim 2 wherein a second ligand is a heteroantibody or reactive fragment thereof.
- 25 5. The method of claim 2 wherein a second ligand is interleukin-2 or a reactive fragment thereof.
- 30 6. The method of claim 1 determining said reactivity enzymatically, radioisotopically, spectrophotometrically, immunologically, fluorescentphotometrically or a combination thereof.
- 35 7. The method of claim 1 determining said reactivity by a competition assay employing radiolabeled and unlabeled ligands.
- 40 8. A monoclonal antibody having specific binding affinity to interleukin-2 receptor at a site different from interleukin-2 binding site derived from a hybridoma having American Type Culture Collection, Accession n° HB8784.
- 45 9. A method of detecting immune activation in humans comprising detecting the presence of soluble interleukin-2 receptor in body fluid by the method according to anyone of claims 1 to 7.
- 50 10. The method of claim 9 wherein said immune activation is that of the lymphoreticular system.
- 55 11. A kit for assaying interleukin-2 receptor (IL-2R) comprising a first container containing a ligand which binds to IL-2R, said ligand consisting of a monoclonal antibody derived from a hybridoma having American Type Culture Collection, Accession n° HB8784 ; a second container having a different ligand also binding to IL-2R, said different ligand being detectable by a method selected from the group consisting of enzymatic, radioisotopic, spectrophotometric, immunologic, fluorescentphotometric and a combination thereof ; and instructions to perform the assay.

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ELISA ASSAY FOR SOLUBLE
HUMAN IL-2 RECEPTORS

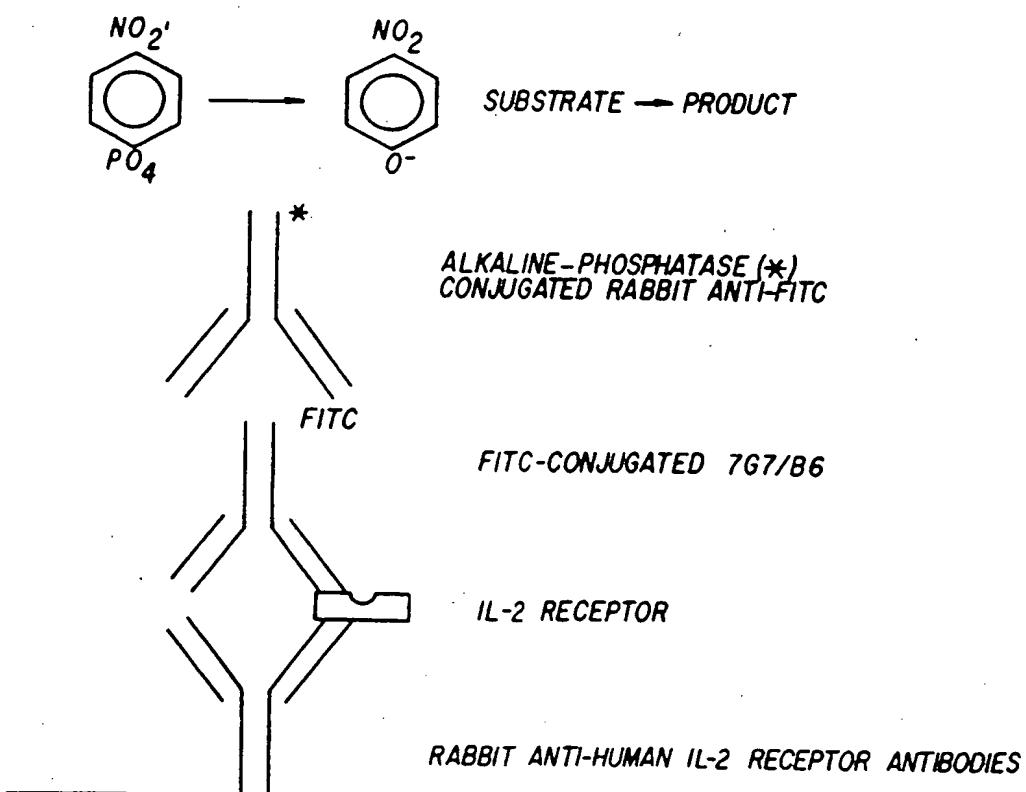


FIG. 1

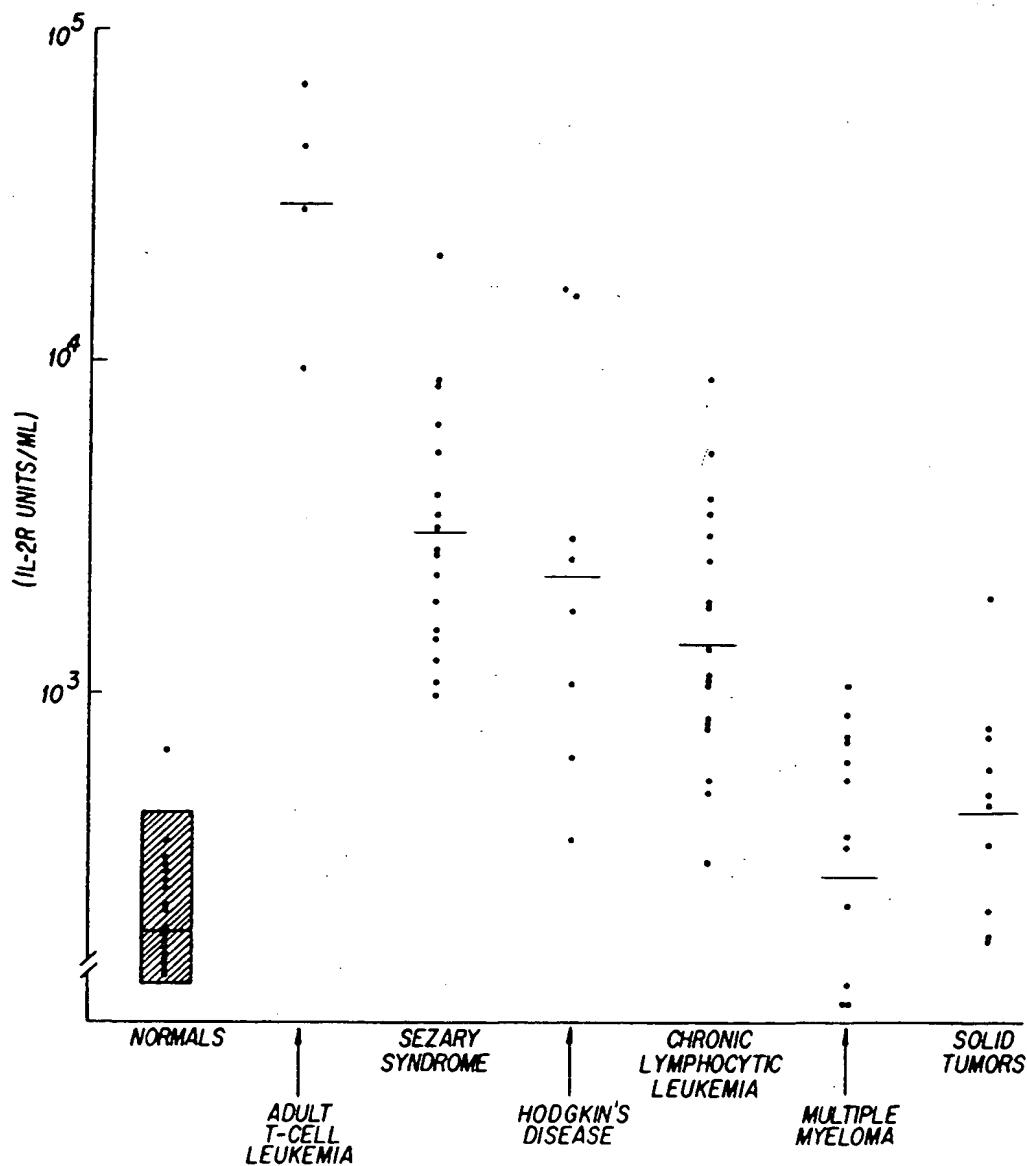
SERUM IL-2 RECEPTOR LEVELS IN NORMALS
AND PATIENTS WITH MALIGNANT DISEASES

FIG. 2

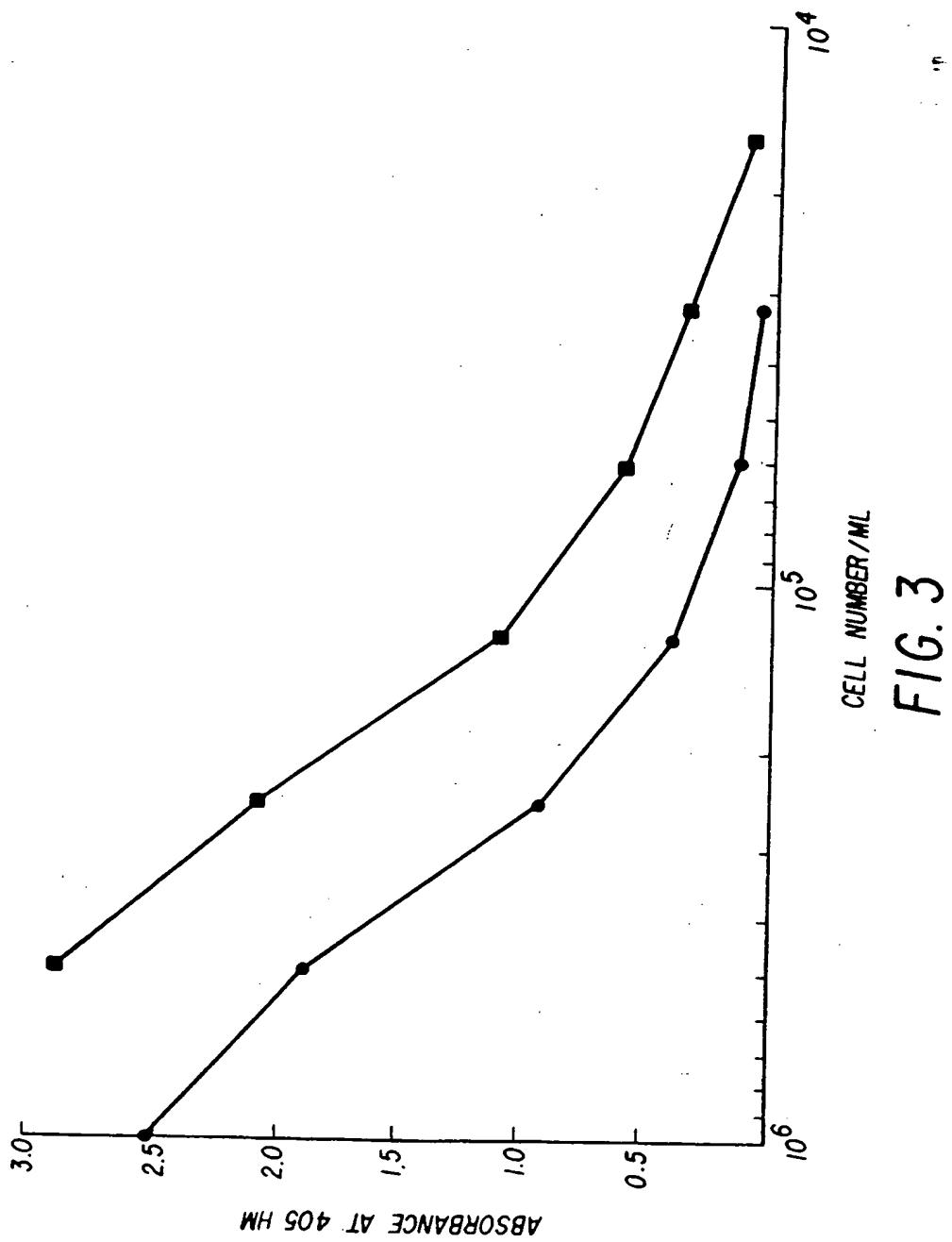


FIG. 3

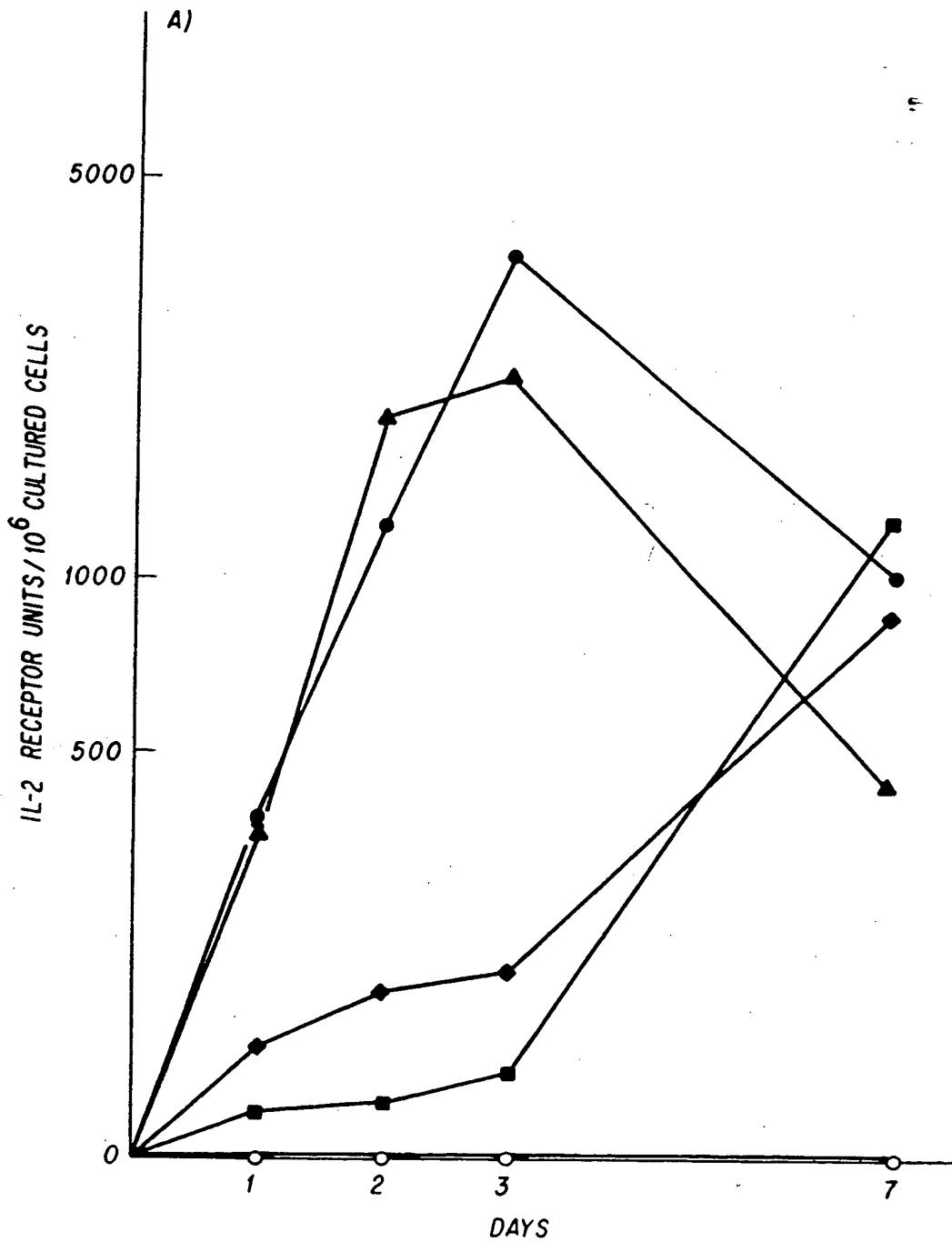


FIG. 4A

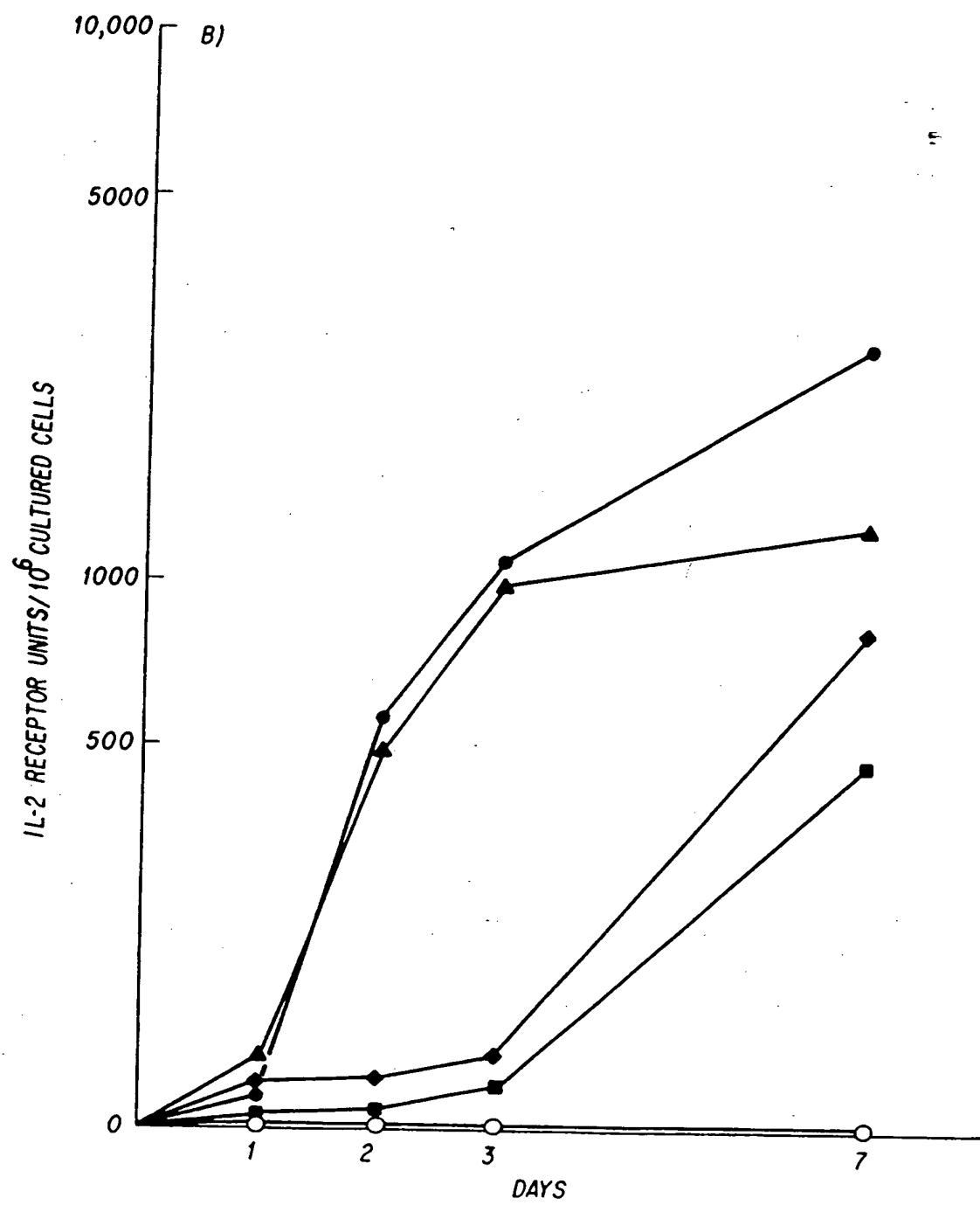


FIG. 4B

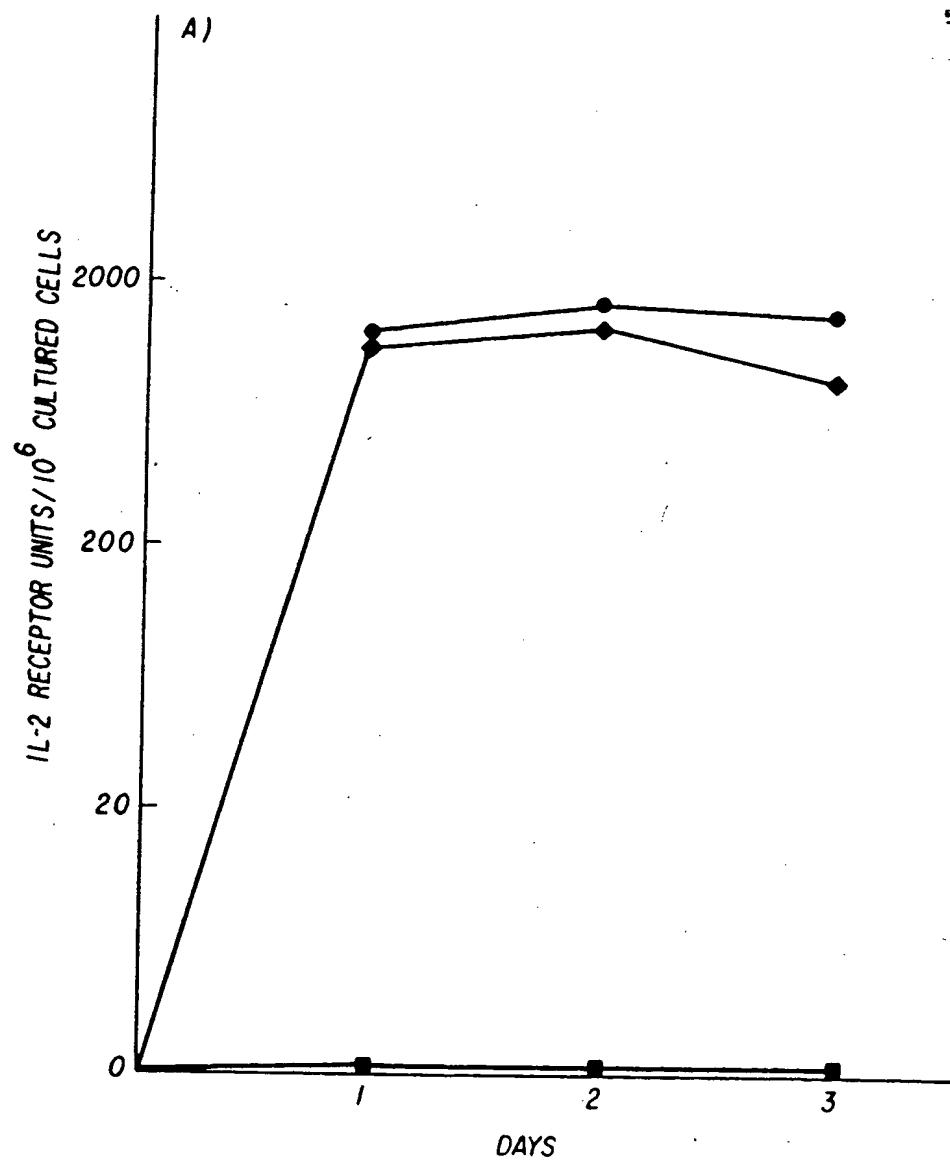


FIG. 5A

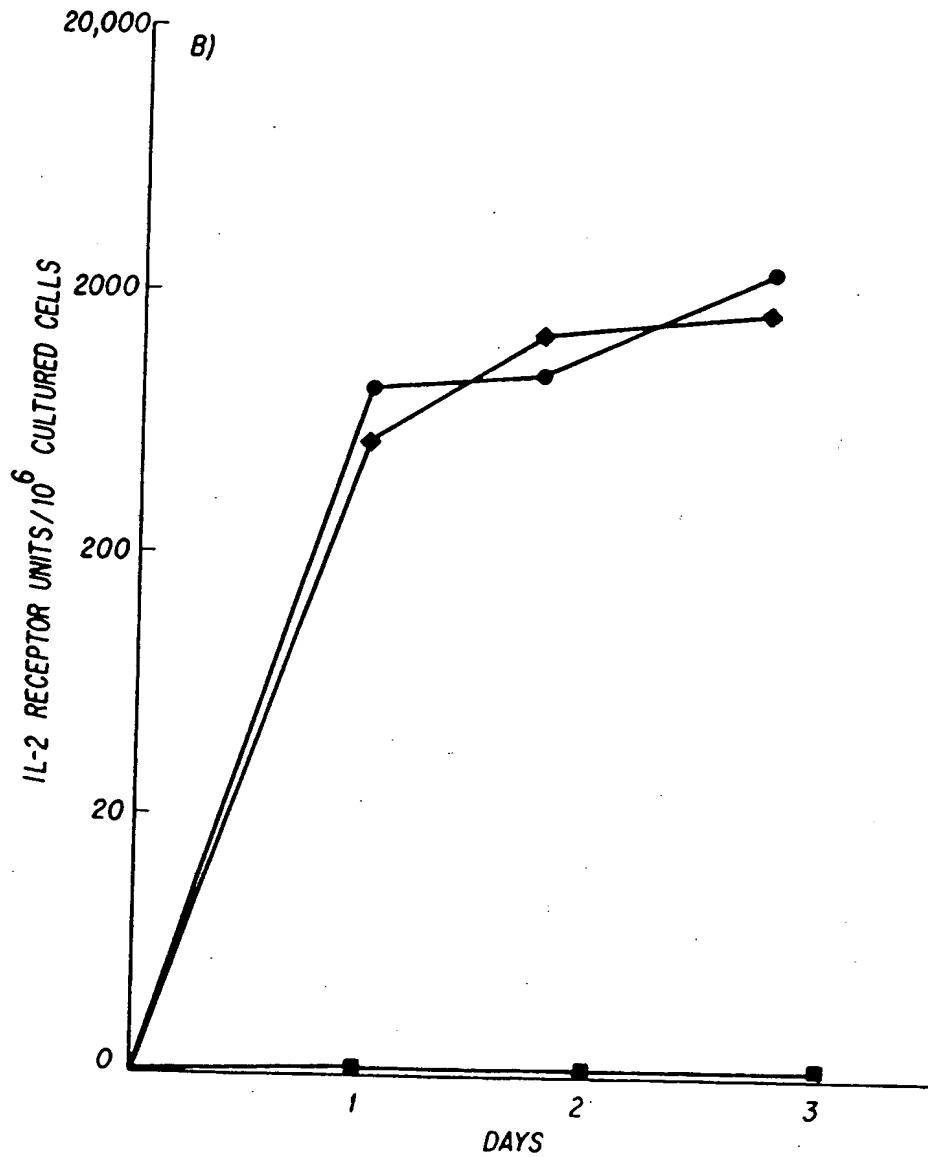
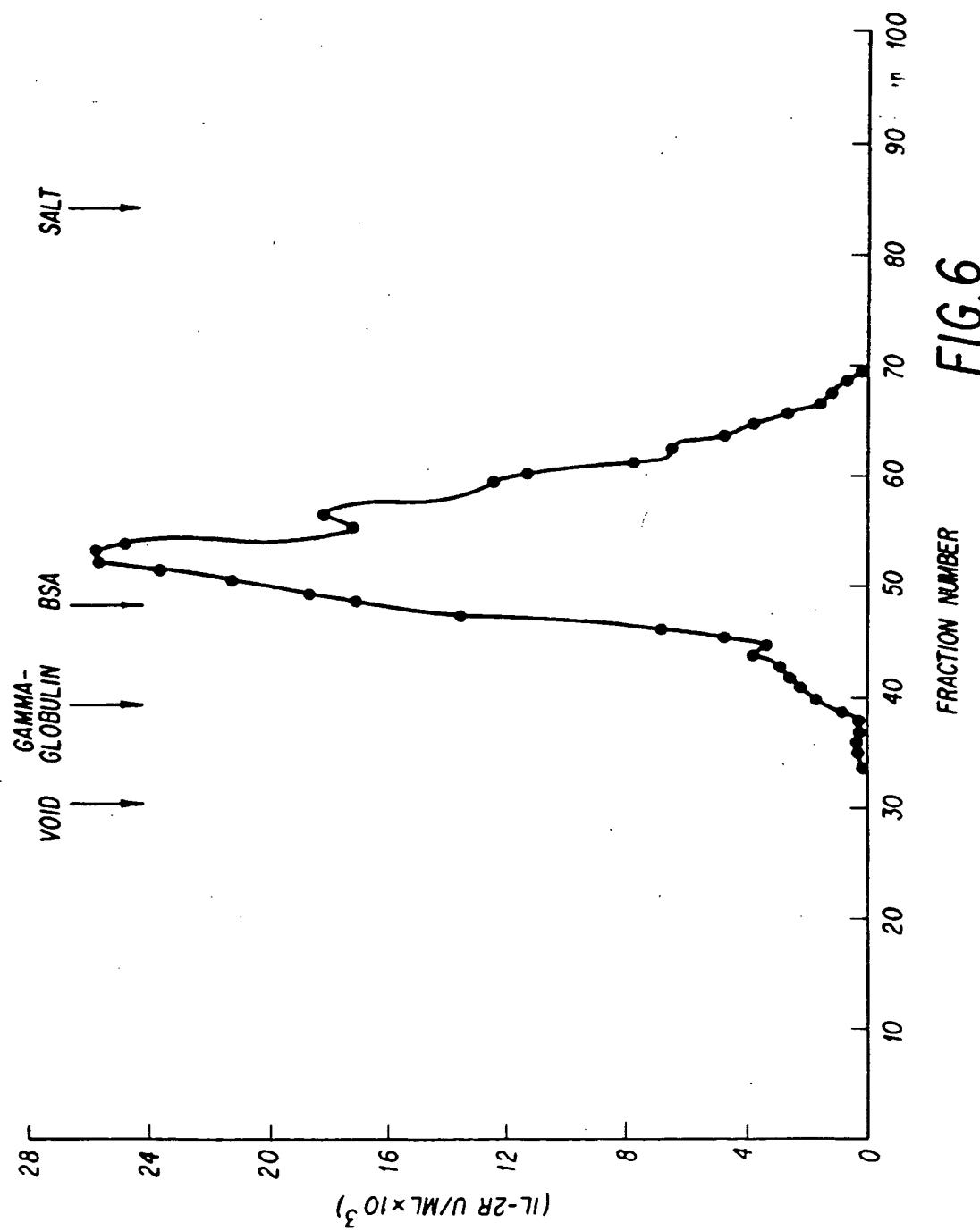


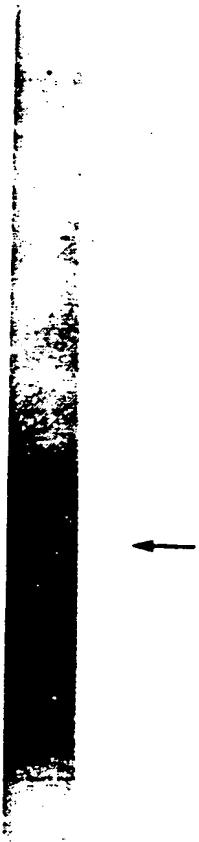
FIG. 5B



SOLUBLE INTERLEUKIN-2 RECEPTOR (HUT 102/B2)

$M_r \times 10^{-3}$

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FIG. 7